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Cloning of Plasmid DNA Sequences Involved in Invasion of HeLa Cells by *Shigella flexneri*

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A large plasmid is found in virulent isolates of *Shigella* sp. and encodes functions essential for invasion of mammalian cells. To identify plasmid sequences necessary for invasion, we isolated a series of Tn*5* insertions in pWR100, the virulence plasmid of *Shigella flexneri* serotype 5. These insertions demonstrated that three separate *Eco*RI fragments of pWR100 were required for invasion of HeLa cells. However, the corresponding native *Eco*RI fragments, when cloned into pBR325, did not restore virulence to plasmidless strains. Construction of a λ-sensitive, plasmidless *Shigella* recipient enabled us to shotgun clone plasmid DNA directly into *S. flexneri* by using the cosmid vector pJB8 and score for expression of invasive functions. In this fashion, we succeeded in isolating six independent recombinants which restored invasion of HeLa cells in plasmidless *Shigella* recipients. The cloned inserts all contained a common core of ca. 37 kilobases, thus defining a minimum sequence necessary for invasion of HeLa cells. Virulence-associated peptides produced by wild-type *S. flexneri* were also produced by the recombinants. Expression of these peptides and expression of invasiveness by the clones were regulated by growth temperature, as is expression of these traits in wild-type *S. flexneri*. A complete invasive phenotype was not expressed by the recombinants in that they failed to produce a positive Sereny test. Possible explanations for this behavior as it relates to the mechanism of bacterial invasion are discussed.

The pathogenic potential of bacteria belonging to the genus *Shigella* is correlated with the ability of these organisms to penetrate and multiply within cells of the human colonic epithelium (16). Commonly used experimental models for measuring the invasive capacity of *Shigella* sp. include the Sereny test, which detects the ability of virulent bacteria to elicit keratoconjunctivitis in the guinea pig (26), and in vitro infection of mammalian cells in tissue culture (16). Through the use of these assay systems, it has been demonstrated that a large plasmid is associated with the invasive phenotype (24, 25). This plasmid ranges in size from 120 to 140 megadaltons (Mdal) and is found in the four *Shigella* species (23). Spontaneous variants which have lost this plasmid are no longer invasive. Restoration of the complete invasive phenotype is accomplished by transfer of the virulence-associated plasmid back into the plasmid-cured mutant (24, 25). pWR100, the 140-Mdal plasmid of *Shigella flexneri* serotype 5, has also recently been shown to encode expression of membrane peptides which are not produced in avirulent mutants (12). Expression of these peptides is thermoregulated, as is expression of the invasive phenotype itself (19; T. L. Hale et al., manuscript in preparation).

Although chromosomal loci are also required for expression of a complete virulence phenotype (9, 22), the role of this plasmid in expression of virulence is certainly a critical one and deserves further genetic and molecular study. Moreover, the high level of homology observed among virulence plasmids belonging to different *Shigella* species, as well as "shigella-like" enteroinvasive *Escherichia coli* (12, 23), should allow us to extend the results of our studies to these other dysentery-producing organisms.

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In this report, we describe the strategy utilized to clone and identify those DNA sequences involved in the invasion of HeLa cells by *S. flexneri*. We found that a plasmid sequence of ca. 37 kilobases (kb) is sufficient to enable an avirulent, plasmidless mutant to invade HeLa cells. Peptides encoded by this cloned DNA fragment react with an antiserum specific for peptides of virulent shigellae and are similar in size to those peptides previously identified as being encoded by the virulence plasmid (12). Strains carrying this cloned plasmid sequence did not, however, express the full spectrum of wild-type invasive properties in that they were unable to produce keratoconjunctivitis in the Sereny test. These results are discussed as they relate to possible mechanisms by which *Shigella* spp. enter and multiply within mammalian cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in L broth (17) or tryptic soy broth (Institut Pasteur Production, Marnes La Coquette, France). For infection with in vitro-packaged cosmid DNA, BS169 was grown in L broth (no glucose) plus 10 mM $MgSO_4$. Fermentation of lactose was scored on MacConkey lactose agar (Difco Laboratories, Detroit, Mich.). Antibiotics were added to broth culture or brain heart infusion (Difco Laboratories) agar in the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml.

Insertion mutagenesis of pWR100. Random insertions of the kanamycin resistance (Km^r)-encoding transposon Tn*5* were generated in pWR100 by use of the replication thermosensitive vector F'(ts114)Lac^+Tn*5* previously described (24). Km^r, lactose-negative mutants were subsequently screened for loss of virulence functions, and the

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Virulence Plasmid	Invasiveness in HeLa cells[b]	Source or reference
S. flexneri 2a				
2457T	Mal$^-$ λ^r	+	+	S. Formal (8)
BS103	Mal$^-$ λ^r	−	−	Spontaneous from 2457T (20)
BS109	Mal$^-$ λ^r *galU*::Tn*10*	+	*	P1L4 transduction of 2457T
BS167	Mal$^+$ λ^s *galU*::Tn*10*	+	*	Spontaneous Mal$^+$ derivative of BS109
BS168	Mal$^+$ λ^r	−	−	Spontaneous Mal$^+$ derivative of BS103
BS169	Mal$^+$ λ^s *galU*::Tn*10*	−	−	P1L4 transduction of BS168
S. flexneri 5				
M90T	Mal$^-$ λ^r	+	+	(25)
M90T-A	Mal$^-$ λ^r	−	−	Spontaneous from M90T
E. coli HB101	F$^-$ *ara-14 leu proA2 lacY1 glnV44* λ^- *galK2 recA13 rpsL20 xyl-5 mtl thi hsdS20*			(3)
pWR100	Virulence plasmid from *S. flexneri* 5 strain M90T			(25)
pJB8	Cosmid cloning vector; Ap^r			(13)
pBR325	Cloning vector; Ap^r Tc^r			(2)
pRZ102	Tn*5* probe			(14)

[a] Genotypes are presented according to the nomenclature of Bachmann and Low (1).
[b] +, Invasive; −, noninvasive; *, bacteria invade HeLa cells and rapidly casue detachment of monolayer.

presence of Tn*5* on pWR100 was verified by hybridization with a ^{32}P-labeled Tn*5* probe as described below.

Virulence assays. The virulence properties of *S. flexneri* were measured by the Sereny test (26) and HeLa cell invasion (11). In the Sereny test, virulent (invasive) *Shigella* strains produced keratoconjunctivitis within 3 days of challenge. More than 95% of the HeLa cells in a challenged tissue culture monolayer were invaded by virulent strains in the standard assay. The plaque assay of Oaks et al. (20a) was also employed as a measure of *S. flexneri* invasiveness of HeLa cells.

Isolation and characterization of plasmid DNA. For rapid screening of plasmid DNA, 1.0-ml overnight broth cultures were extracted by the technique of Kado and Liu (15). DNA for restriction analysis and hybridization was isolated by a modification of this technique (12) and purified by centrifugation through a cesium chloride-ethidium bromide density gradient.

Restriction endonuclease digestion of plasmid DNA was performed according to the recommendations of the manufacturers. Agarose gel electrophoresis of intact and restricted plasmid DNA was run in 0.7% vertical gels with E buffer (40 mM Tris, 2 mM disodium EDTA [pH 7.9]) or Tris borate (89 mM Tris base, 2.5 mM disodium EDTA, 89 mM boric acid [pH 8.0]), respectively, as running buffers.

Cosmid in vitro packaging and other cloning techniques. The methods used for constructing and manipulating recombinant DNA molecules were essentially those described by Maniatis et al. (18). Plasmid pWR100 DNA was partially restricted with *Sau*3A and sized in an NaCl gradient. Cosmid vector pJB8 (13) was cleaved with *Bam*HI and dephosphorylated. Donor and vector DNA were ligated at high concentrations to produce concatemers. Ligated, recombinant molecules were packaged into phage heads by using a packaging mix kindly provided by Colin Bishop, Institut Pasteur. The resulting cosmid lysate was used to transduce BS169 with selection for ampicillin resistance, the selective marker of the cosmid vector.

The cloning vector pBR325 (2) was used for subcloning *Eco*RI fragments of pWR100 and its derivatives. Transformation of plasmid DNA into *E. coli* HB101 or *Shigella* recipients was performed as previously described (7).

Preparation of DNA probes and hybridization. Tn*5* probe DNA was prepared from pRZ102 by restriction with *Hin*dIII and extraction of the 3.2-kb internal Tn*5* fragment from a 0.7% low melting point agarose gel (Bethesda Research Laboratories, Gaithersburg, Md.). *Eco*RI fragment probes from pWR100 were prepared in a similar fashion (18). DNA probes were labeled with ^{32}P (Amersham Corp., Buckinghamshire, England) by nick translation (21). DNA fragments were transferred from agarose gels to nitrocellulose filters (Schleicher & Schull, Inc., Dassel, Federal Republic of Germany), and hybridizations were carried out by the method of Southern (27).

Western blot hybridization analysis. Whole bacterial extracts were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and blotted onto nitrocellulose filters as described by Burnette (4). Diluted (1:250) serum from a

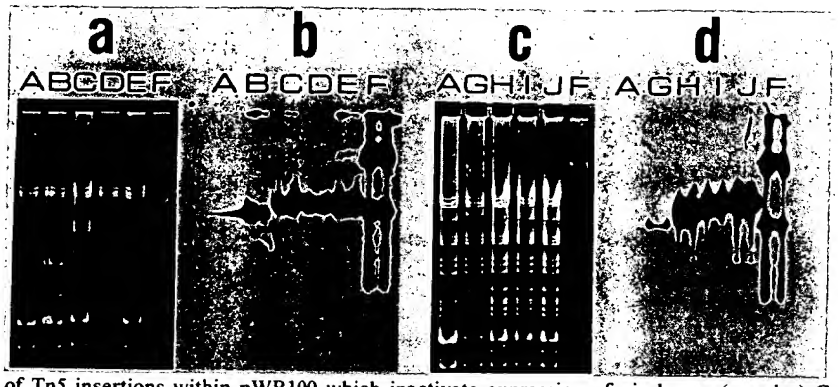

FIG. 1. Localization of Tn*5* insertions within pWR100 which inactivate expression of virulence. (a and c) Gel photographs of *Eco*RI digestion patterns of pWR100::Tn*5* derivatives. (b and d) autoradiographs of the same gels shown in panels a and c after Southern transfer and hybridization with a ^{32}P-labeled Tn*5* probe. Lanes: A, pWR100; B, pHS1042; C, pHS1059; D, pHS1060; E, pHS1095; F, nonrestricted pRZ102 (Tn*5* probe); G, pHS1304; H, pHS1345; I, pHS1378; J, pHS1389.

monkey immunized against *S. flexneri*, which specifically recognized four major peptides encoded by the virulence plasmid of M90T (Hale et al., manuscript in preparation), was used to detect expression of these peptides by the recombinant clones.

Strain construction. Standard genetic techniques for generalized transduction by P1L4 and selection for spontaneous maltose-fermenting (Mal$^+$) variants of *S. flexneri* were employed (6).

RESULTS

Insertion mutagenesis of pWR100 and identification of sequences involved in HeLa cell invasion. To identify regions on pWR100 which were essential for invasion of HeLa cells, random insertions of Tn*5* were isolated in the plasmid by the use of the Tn*5* donor, F′(ts114)Lac$^+$Tn*5*. Approximately 1,000 Km^r mutants were screened for the ability to penetrate HeLa cells. In eight noninvasive mutants identified, no deletion of plasmid DNA could be detected, and transposition of Tn*5* onto pWR100 was verified by hybridization with a ^{32}P-labeled Tn*5* probe (Fig. 1).

These plasmids were referred to as pHS1042, pHS1059, pHS1060, pHS1095, pHS1304, pHS1345, pHS1378, and pHS1389. The restriction enzyme *Eco*RI, which has no cleavage site within Tn*5* (14), was used to characterize these plasmids. An increase in fragment size of 5.4 kb (corresponding to the size of Tn*5*) and a positive signal of the newly generated fragment after hybridization with the Tn*5* probe demonstrated that Tn*5* had inserted into *Eco*RI fragments of pWR100 of three different sizes: 7.6, 11.5, and 17 kb (Fig. 1). Tn*5* had inserted within the 7.6-kb fragment in pHS1042, the 11.5-kb fragment in pHS1059, pHS1060, pHS1095, pHS1304, and pHS1345, and the 17-kb fragment in pHS1378 and pHS1389. The corresponding, unmutagenized *Eco*RI fragments from the parent plasmid, pWR100, were subcloned into the *Eco*RI site of the cloning vector pBR325. The resultant recombinant plasmids were transformed into an avirulent, plasmid-cured *S. flexneri* strain, M90T-A, and assayed for invasiveness in HeLa cells. None of the cloned parental *Eco*RI fragments were capable of restoring invasiveness to strain M90T-A.

Cosmid cloning of pWR100 virulence sequences. The technique of cosmid cloning required construction of a λ-sensitive, plasmid-cured *Shigella* recipient which could be screened for expression of the invasive phenotype in HeLa cells. Genealogy and properties of such a strain, BS169, and an invasive, isogenic control strain, BS167, are listed in Table 1. A spontaneous, noninvasive derivative of *S. flexneri* 2a was isolated. This strain, BS103, had lost the 140-Mdal virulence plasmid (20). Since Mal$^+$ shigellae are capable of expressing the λ receptor and are sensitive to host range mutants of λ (10), a spontaneous Mal$^+$ derivative of BS103 was next isolated. Introduction of the *galU*::Tn*10* mutation into a Mal$^+$ *S. flexneri* strain renders the strain sensitive to wild-type λ, and such a strain can then be used as a recipient for cosmid cloning. An additional advantage of the *galU*::Tn*10* mutation is that, in a virulent background, it alters the virulence properties of the bacteria such that invasion of HeLa cells causes the monolayer to detach 1 to 2 h after infection (A. T. Maurelli and R. Curtiss III, manuscript in preparation). Isogenic *galU*::Tn*10* *S. flexneri* strains which do not possess the 140-Mdal virulence plasmid and cannot invade the cells have no deleterious effect on the monolayer.

Plasmid pWR100 DNA was cloned into the cosmid vector pJB8, packaged into λ heads, and transduced into BS169. A bank of 800 transductants was screened for clones capable of causing monolayer detachment of HeLa cells. Six stable recombinant plasmids capable of restoring invasiveness to BS169 were identified. Their sizes and the virulence phenotypes of these recombinant plasmids transformed into strain M90T-A are summarized in Table 2. Temperature-dependent

TABLE 2. Characterization of clones containing cosmid recombinant molecules

Strain	% HeLa cells invaded when bacteria were grown at:		Plaque assay	Sereny test	Size of recombinant plasmids (kb)
	30°C	37°C			
M90T	<1	95	+	+	
M90T-A	0	0	−	−	
M90T-A(pHS4108)	5	49.2	−	−	49.2
M90T-A(pHS4181)	5	26	−	−	42
M90T-A(pHS4195)	3	42	−	−	48.5
M90T-A(pHS4685)	1	8.5	−	−	42.9
M90T-A(pHS4707)	2	16	−	−	45
M90T-A(pHS4717)	1	16	−	−	42.9

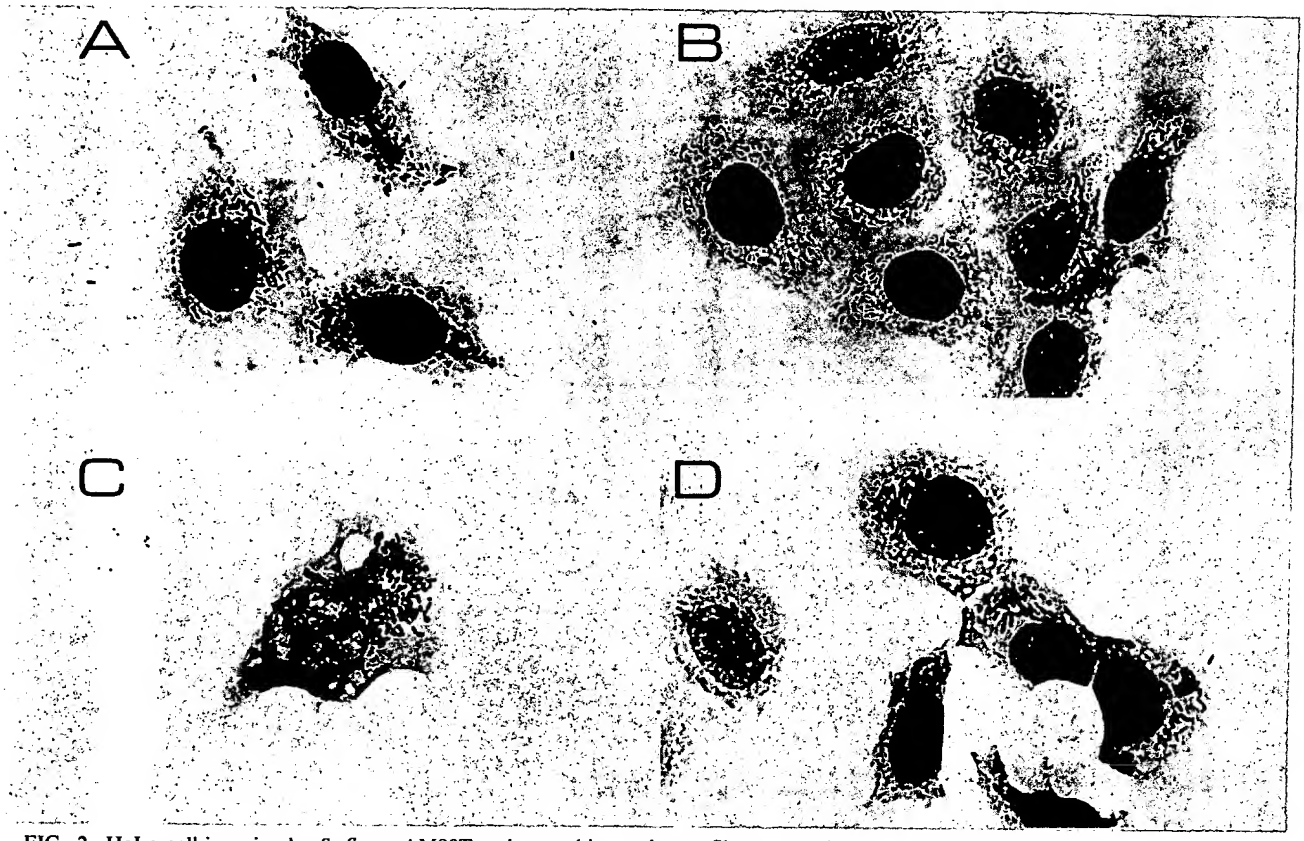

FIG. 2. HeLa cell invasion by *S. flexneri* M90T and recombinant clones. Shown are photomicrographs (×100) of HeLa cells challenged by *S. flexneri* grown at 37°C. (A) M90T; (B) M90T-A; (C) BS169(pHS4108); (D) M90T-A(pHS4108).

expression of invasiveness, which was recently described in *Shigella* sp. (19), was also conserved in the recombinants. An example of restoration of the HeLa cell invasive phenotype by recombinant plasmid pHS4108 is compared in Fig. 2 with that of wild-type strain M90T. Strain M90T-A carrying pHS4108 did not express a full pattern of virulence since only half the cells of the monolayer were invaded. In addition, the cells were not invaded as heavily and diffusely as those infected by M90T.

This difference in the rate and intensity of HeLa cell invasion by strain M90T-A carrying the recombinant plasmids was also reflected in the plaque assay. When an infected monolayer is overlaid with culture medium plus agarose supplemented with 20 μg of gentamicin per ml, which kills extracellular bacteria, the invasive microorganisms penetrate and multiply freely within the HeLa cells, invade contiguous cells, and finally produce a cytotoxic effect resulting in a plaque (20a). In the plaque assay, M90T and BS167 appeared positive, whereas BS169 and M90T-A, both carrying pHS4108, were consistently negative. The other recombinant plasmids, transformed into strain M90T-A, also failed to produce plaques on HeLa cells. These same strains were negative in the Sereny test as well (Table 2).

Restriction and homology of recombinant plasmids. The six recombinant clones which restored HeLa cell invasiveness in BS169 were examined in homology with regions of pWR100 known to be essential for virulence. Tn*5* insertions into pWR100 *Eco*RI fragments of 7.6, 11.5, and 17 kb had earlier been shown to abolish expression of virulence in *S. flexneri* (see above). We therefore utilized the corresponding unmutagenized *Eco*RI fragments (cloned into pBR325) as hybridization probes to detect their presence in the recombinant plasmids. Figure 3 shows the *Eco*RI restriction patterns of the recombinant plasmids and hybridization with the 7.6-, 11.5-, and 17-kb *Eco*RI fragments of pWR100. All six clones hybridized with the three *Eco*RI fragment probes, thus demonstrating the presence of parts or all of these sequences and confirming their association with invasiveness. A physical map of the recombinant plasmids, constructed by use of *Eco*RI and *Sal*I restriction endonucleases, showed them to be similar in size with different endpoints (Fig. 4). Both the 7.6- and 11.5-kb *Eco*RI fragments were completely contained within each clone and at least 9 kb of the 17-kb fragment was present in each as well. Thus, a provisional minimum sequence of 37 kb was defined as necessary for HeLa cell invasion.

In an attempt to locate the sites of Tn*5* insertions within the virulence plasmid which abolished invasiveness, pWR100::Tn*5* insertion derivatives were cleaved with *Eco*RI and cloned into pBR325. The location of the Tn*5* insert within each *Eco*RI fragment was mapped through the use of several different restriction enzymes. Localization of these transposons is indicated in Fig. 4 on the map of clone pHS4108. The six insertions mapped in two regions: a single insertion lay at one end of the cloned DNA within a 1.7-kb *Bam*HI fragment, and a cluster of insertions lay at the opposite end within a 9.3-kb *Sal*I-*Xho*I sequence.

Western blotting of peptides expressed by recombinants. To demonstrate that the recombinant clones produced peptides which were associated with expression of the invasive

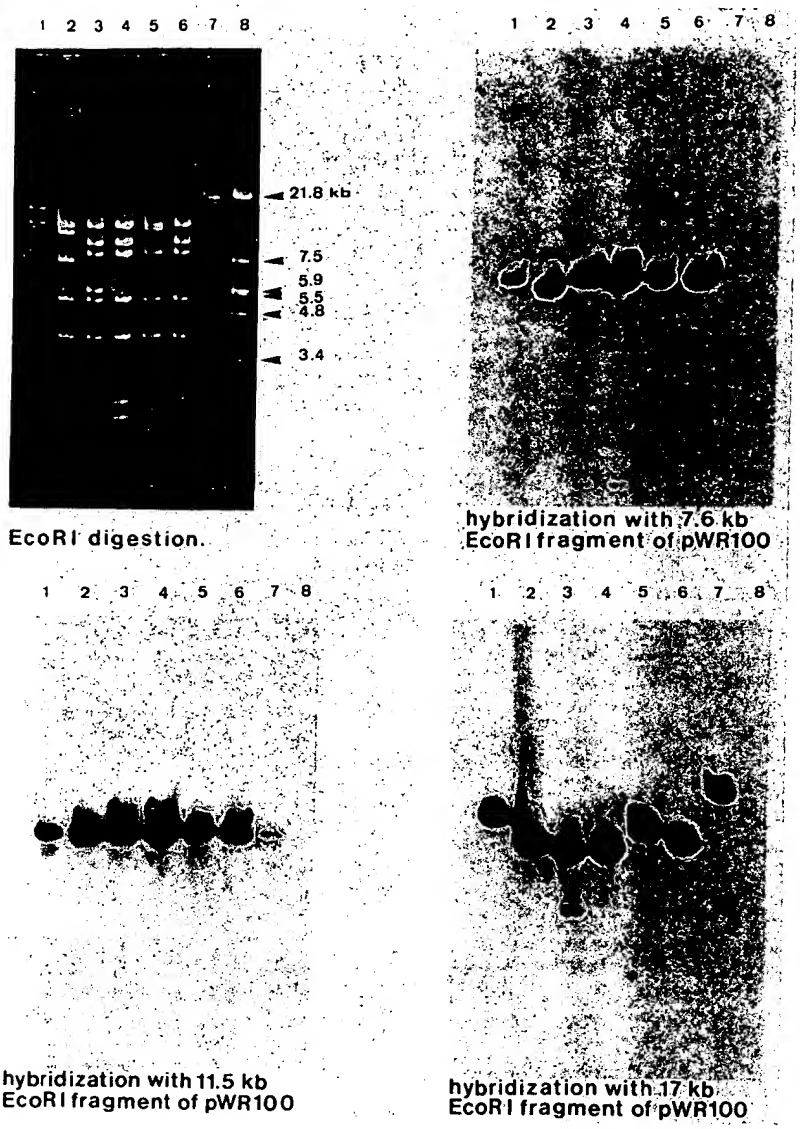

FIG. 3. *Eco*RI digestion of recombinant plasmids and hybridization with ^{32}P-labeled *Eco*RI fragments of pWR100. Lanes: 1, pHS4108; 2, pHS4181; 3, pHS4195; 4, pHS4685; 5, pHS4707; 6, pHS4717; 7, pWR100; 8, phage λ DNA.

phenotype by the parent M90T, Western blot analysis of whole bacterial extracts was performed. The antiserum used had been shown to specifically recognize four major peptides in extracts of whole bacteria carrying pWR100 (Hale et al., manuscript in preparation). These same four peptides were not produced by strain M90T-A, a plasmid-cured, avirulent derivative of strain M90T.

As seen in Fig. 5, peptides of the same molecular weight as peptides a, b, c, and d of strain M90T were expressed by recombinant clones pHS4108, pHS4181, and pHS4195 and recognized by the specific antiserum to these virulence-associated peptides. The clones produced relatively the same amount of each peptide as strain M90T, except for peptide d which was reduced in amount in each clone. Strain M90T grown at 30°C produced very little detectable amounts of these peptides, which was consistent with repression of the invasive phenotype at this temperature. In contrast, however, when recombinant clone pHS4108 was grown at 30°C, it reproducibly expressed more peptides b and c than did strain M90T at this temperature. Invasion of HeLa cells by strain M90T-A carrying pHS4108 was also slightly derepressed at 30°C (Table 2).

DISCUSSION

The invasion of epithelial cells of the large intestine is an essential feature of the pathogenicity of dysentery bacilli and requires the expression of genes located on a large plasmid. Genetic and physical analysis of the virulence-associated plasmids of *Shigella* sp. has proved difficult because of their large size (120 to 140 Mdal) and relative instability. In addition, deletions of the plasmid in *S. flexneri*, which reduce its size by 20 to 95 Mdal, also cause loss of the ability to penetrate mammalian cells (20). Therefore, we undertook to study expression of genes from pWR100, the virulence plasmid of *S. flexneri* serotype 5, by recombinant DNA techniques.

Using Tn*5* insertion mutagenesis, we were first able to identify three different *Eco*RI fragments from pWR100

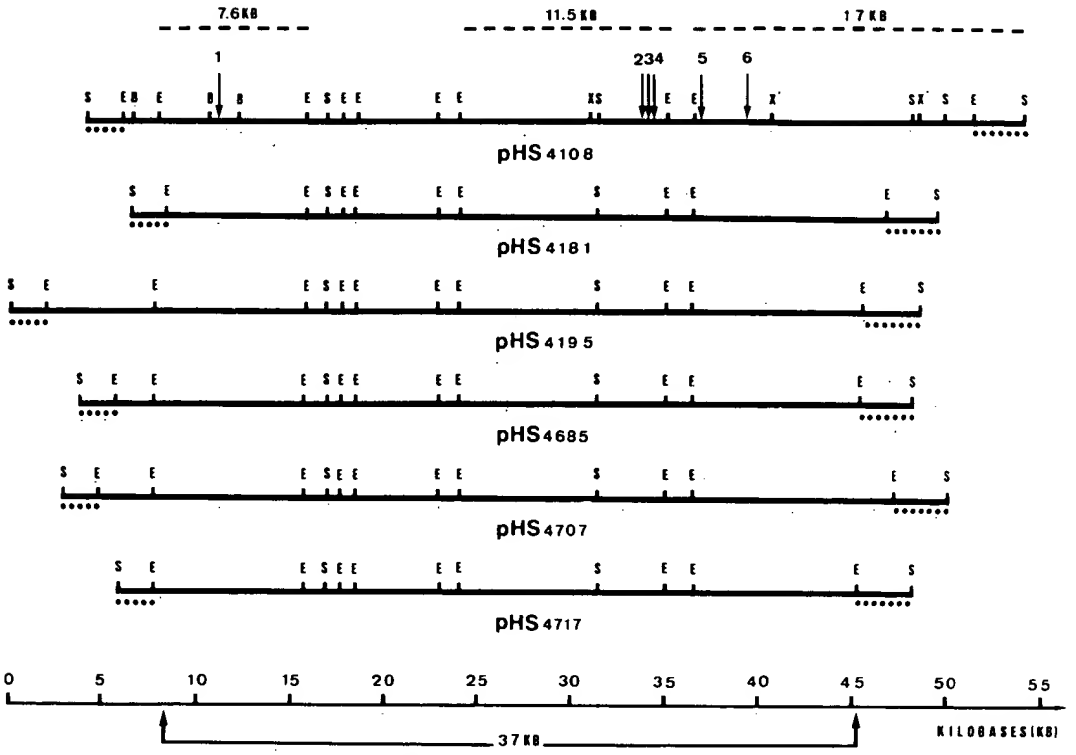

FIG. 4. Physical maps of recombinant plasmids which restored invasion of HeLa cells. E, B, X, X', and S represent the restriction sites of *Eco*RI, *Bam*HI, *Xba*I, *Xho*I, and *Sal*I, respectively. Mapping of restriction sites with *Bam*HI, *Xba*I, and *Xho*I was done only for pHS4108. Dotted lines below each plasmid indicate the DNA sequences of the cosmid cloning vector, pJB8. Sequences corresponding to the three *Eco*RI fragments of pWR100 in which Tn*5* insertions inactivated virulence are indicated above the map of pHS4108. Map positions of six independent Tn*5* insertions in pWR100 which inactivated virulence are shown by numbered arrows on pHS4108 and correspond to plasmids pHS1042, pHS1059, pHS1060, pHS1095, pHS1378, and pHS1389, respectively.

which encoded functions necessary for invasion of HeLa cells. Since none of the native *Eco*RI fragments alone restored invasiveness to strain M90T-A, we concluded that a larger sequence of plasmid DNA was needed for expression of the invasive phenotype. To improve the likelihood of cloning sequences of pWR100 large enough to encode all the functions necessary for invasion of HeLa cells, a cosmid vector was used since cosmids permit cloning of large (up to 50 kb) fragments of DNA (5). The construction of strain BS169 enabled us to transduce the cosmid lysate directly into a plasmidless *Shigella* recipient, thereby eliminating the need for an *E. coli* intermediate. In addition, the *galU* mutation in BS169 greatly facilitated screening of the cosmid library for clones which restored invasiveness, since the monolayer detachment assay was simpler and faster to score than microscopic visualization of monolayers for invaded cells.

FIG. 5. Western blot hybridization analysis of peptides expressed by recombinant clones. Recombinant plasmids were transformed into strain M90T-A for analysis of expression of peptides encoded by the cloned insert. Strains were grown at 30 or 37°C as indicated. Lanes: 1, M90T at 37°C; 2, M90T-A at 37°C; 3, M90T at 30°C; 4, pHS4108 at 37°C; 5, pHS4108 at 30°C; 6, pHS4181 at 37°C; 7, pHS4195 at 37°C. Molecular weights of peptides a, b, c, and d are approximate. MOMP is a major outer membrane protein unrelated to virulence.

Cosmid cloning of pWR100 yielded six independent recombinants which were capable of restoring invasiveness to strain M90T-A for HeLa cells. Each of these clones was found to contain the three *Eco*RI fragments earlier identified by Tn*5* mutagenesis, thus confirming their importance in expression of virulence. These *Eco*RI fragments were not, however, contiguous. Restriction mapping demonstrated that a common core sequence of ca. 37 kb was present in each clone, thus defining a minimum sequence necessary for expression of the invasive phenotype. Fine mapping of specific Tn*5* insertions which abolished virulence showed that the insertions clustered at two widely separated regions: the 7.6-kb leftward *Eco*RI fragment and the 16.1-kb rightward *Sal*I fragment (Fig. 4). This clustering may reflect the presence of virulence genes at opposite ends of the cloned plasmid sequence with large, noncoding or nonvirulence-related sequences in between. On the other hand, a large sequence of plasmid DNA may indeed be essential for expression of virulence.

It was of particular interest that the expression of invasiveness by the recombinants remained subject to control by growth temperature. This suggested that we had also cloned a regulatory mechanism which responds to temperature and thereby modulates expression of virulence genes on the plasmid. The temperature-dependent expression of virulence-associated polypeptides from the recombinants also supports this interpretation. Recombinant pHS4108 did express slightly elevated levels of peptides b and c when grown at 30°C, and it also was slightly invasive at this temperature, in contrast to strain M90T. The residual expression of virulence functions by pHS4108 at 30°C may be a reflection of a higher gene dosage of the cloned insert, since cosmid vectors are maintained as multicopy plasmids in the host bacteria (5).

The Western blot analysis demonstrated that at least four virulence plasmid-specific peptides are produced by recombinant plasmids pHS4108, pHS4181, and pHS4195. These results further strengthen the association of these peptides with expression of the virulence phenotype. We are currently constructing a more detailed genetic and physical map of pHS4108 through the use of Tn*5* insertions to determine the location of coding sequences for these peptides in the cloned DNA and to more precisely demonstrate their function in the invasion of HeLa cells by *S. flexneri*. Preliminary evidence would appear to indicate that some Tn*5* insertions to the right of the 7.6-kb *Eco*RI fragment of pHS4108 abolish invasiveness and also block expression of these four virulence-associated peptides.

Although we have been able to restore temperature-regulated invasion of HeLa cells with the recombinant plasmids, the clones failed to produce a positive Sereny test or plaque assay. Formation of plaques is likely to be dependent on the bacteria being able to multiply freely within the cytoplasm of infected cells, invade adjacent cells, and lyse the cells. This cycle of invasion, release, and invasion produces the foci of lysed cells which results in a plaque. The negative response of the recombinants in the plaque assay may be due to one of two possibilities: either we have not cloned all of the sequences necessary for expression of the complete invasive phenotype, or cloned virulence genes are not optimally expressed by the recombinant plasmids. Preliminary electron microscopic studies of infected HeLa cells show a difference in the time course and efficiency of disruption of vacuolar membranes of phagosomes containing M90T-A(pHS4108) as compared with phagosomes containing the parental strain, M90T (P. Sansonetti, A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier, submitted for publication). Although this observation does not directly favor either hypothesis, it would explain the phenotypic difference, since bacteria are expected to grow more efficiently when free within the cytoplasm as opposed to within a phagosome. Our current efforts are directed towards subcloning coding sequences from recombinant pHS4108 into expression vectors with the goal of improving expression of virulence-associated gene products and identification of their role in the invasion of mammalian cells by *Shigella* spp.

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